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Simultaneous detection of hepatitis c virus antigen and antibodies in dried blood spots

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ABSTRACT

Background: Enzyme immunoassays (EIA) designed to detect hepatitis C virus (HCV) core antigen and anti-HCV antibodies (HCV AgAb) simultaneously can improve the early detection of HCV infection when molecular diagnostic methods are not widely available.
Objectives: To evaluate the suitability of dried blood spot (DBS) samples for detecting HCV AgAb using commercial EIAs.
Study design: Paired serum and DBS samples were assayed using two commercial EIAs for HCV AgAb (Monolisa™ HCV AgAb ULTRA and Murex HCV AgAb). Manufacturer's recommendations were followed for sera while sample volume, incubation time and cut-off (CO) determination were evaluated for the DBS samples. The values of sensitivity, specificity, inter-rater agreement, detection limit, assay precision and stability of DBS samples at different conditions (22–26 °C, 2–8 °C and −20 °C) were determined.
Results: It was necessary to increase the DBS sample volume fourfold compared to the sera samples to approximate the DBS Optical Density (OD) values to the sera OD values. Using ROC curve to recalculate CO values for the DBS samples, sensitivity was 97.5% for both EIAs, while the specificity was 99.71% for Monolisa™ HCV AgAb ULTRA and 95.95% for Murex HCV AgAb. Accurate testing results were obtained with DBS samples for 60 days at all conditions evaluated; storage at −20 °C resulted in low OD variation. Both EIAs demonstrated the same limit of detection among DBS samples [estimated viral load of 3.1 International Units per millilitre (IU/mL)] and low OD value variability in repetitivity and reproducibility studies.
Conclusion: DBS samples can be used for the detection of HCV AgAb by EIA as they present comparable performance characteristics and excellent stability among various storage conditions.

1. Background

Hepatitis C virus (HCV) infection can be diagnosed by the presence of antigen, antibody or viral genome among sera or plasma samples. Three generations of enzyme immunoassays (EIAs) for antibodies against HCV (anti-HCV) have been developed that increased sensitivities from 80% among first generation ELISAs to 98% among third generation ELISAs and that reduced the window period from 82 to 66 days.[1,2] Assays for the simultaneous detection of anti-HCV and HCV core antigen (HCV AgAb) have been developed. These new assays have advantages over conventional assays, including a sensitivity of 100% and a specificity of 99.5%, which allows for earlier diagnosis, especially in immunocompromised patients.[3,4]

In Brazil, the prevalence of HCV varies from 1.12% to 1.64%, and the highest occurrence rates have been observed in the northern region of Brazil.[5] Brazil is a large country, and some areas, especially the regions with the highest HCV occurrence, are far from specialised laboratories. Accordingly, the use of dried blood spots (DBS) would be advantageous over conventional blood sampling because this method would allow for the collection and transportation of samples from remote areas to reference laboratories. Currently, DBS samples have been used for anti-HCV and HCV RNA detection,[6–14] but no study has reported the simultaneous detection of HCV antigen and antibodies in DBS.

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2. Objective

The objectives of this study were to evaluate the possibility of simultaneous detection of anti-HCV and HCV core antigen in DBS using commercial ELISA kits and to assess the stability of DBS stored at diverse conditions (22–26 °C, 2–8 °C and −20 °C).

3. Study design

3.1. Population

In this study, 386 individuals were included. Of the study participants, 40 subjects were anti-HCV/HCV RNA reactive and were referred from viral hepatitis centres in Rio de Janeiro. A total of 346 subjects were anti-HCV/HCV RNA negative and had donated blood samples at two hospitals in Rio de Janeiro (Unimed Hospital and Federal Hospital, from the Servers of the State of Rio de Janeiro – HFSE). The samples were collected from January 2009 to March 2010, and the study was approved by the ethics committees of Fiocruz and HFSE. Most of the study subjects were female (56.2%), and the mean age was 40 years, with the ages ranging from 21 to 60 years.

3.2. Sample collection and preparation

Venous blood was collected into Vacutainer tubes (BD, San Jose, CA, USA). One aliquot of blood was centrifuged to obtain the sera, while 75 μL of whole blood was inoculated onto Whatman filter paper n°903 (GE Healthcare, Piscataway, NJ, USA).

Alternatively, 3–5 drops of capillary blood obtained by a finger prick were inoculated onto the same type of filter paper. The DBS samples were air-dried at room temperature for 4 h, placed in individual sealable plastic bags containing silica desiccant sachets and stored at −20 °C.

A 3 mm disc was punched from each DBS sample and placed into microtubes containing 300 μL of buffer PBS/BSA 0.5% at 4–8 °C for 18–24 h. The blood was then eluted from the paper as described previously.[14,15]

3.3. Enzyme immunoassays

Two enzyme immunoassays were used: the Monolisa™ HCV AgAb ULTRA, Bio-Rad (Marnes-la-Coquette, France), and Murex HCV AgAb, Abbott (Kyalami, Republic of South Africa). The assays detected the HCV antigen and the antibody simultaneously. The sera samples were tested according to the manufacturer's recommendations, with 50 μL of sera used for the EIAs. For the Murex HCV AgAb assay, 50 μL of specimen diluent was used. For the DBS sample, a preliminary panel of ten subjects (5 positive for HCV AgAb and 5 negative for HCV AgAb) donated paired sera and DBS samples to determine the initial DBS sample volume without specimen diluents (50 μL, 100 μL, 150 μL, 200 μL) and the sample incubation time (determined by the manufacturer, with 90 minutes used for the Bio-Rad assay and 60 min used for the Abbott assay, and twofold increasing periods of time).

After establishing these parameters, the cut-off (CO) absorbance values for the DBS specimens were calculated by three methods using the entire population that was studied ($n = 386$). CO1 was defined using the manufacturer's recommendations, and CO2 was defined as 3 standard deviations above the mean DBS absorbance of the HCV seronegative samples (mean + 3 SD). CO3 was the area under the receiver operating characteristic curve (AUROC),[16,17] which was created using MedCalc statistical software (version 9.2.1.0, MedCalc Software, Belgium).

3.4. Determination of the limit of detection, precision and stability of HCV AgAb using DBS

HCV AgAb reactive sera samples (OD values above 3.0 in both EIAs and a viral load equal to 3100 International Units per millilitre (IU/mL)) were serially diluted tenfold in negative whole blood samples (dilution series from 1:10 to $1:10^{10}$), and this dilution was serially inoculated (75 μL) onto Whatman 903 paper. This panel was processed as described in Section 3.2 and tested using the two EIAs after optimisation to determine the limit of detection. The capillary whole blood was obtained by digital punch, and the procedure described above was performed.

The HCV AgAb DBS reactive samples (dilutions 1:10 and 1:1000) were tested 8 times in a single run using both EIAs, and the mean, standard deviation, and coefficient of variation (CV) among the replicates were obtained to determine the precision of the EIAs. Repetitivity was determined by testing a sample 20 times in the same run, with a single technician using the same reagents, lot and operator.[18] Reproducibility was obtained by testing identical samples ten times in a single run on two days using the same lot and reagents but different operators.[18]

Stability was determined using one HCV AgAb reactive DBS sample and one negative DBS sample that were prepared and processed as described in Section 3.2. These samples were stored at 20–25 °C, 2–8 °C and −20 °C for 7, 14, 28, 60 and 117 days.[19] OD values from each day were compared to the initial value (day 7) to determine the recovery of HCV AgAb detection. All assays were performed in triplicate.

3.5. Statistical analysis

HCV AgAb detection in serum samples was used as the gold standard for to assess the sensitivity, specificity, and positive (PPV) and negative (NPV) predictive values of the DBS HCV AgAb assay. The descriptive statistics are shown as the mean ± standard deviation or the median and interquartile range, as appropriate. The categorical variables were compared between groups by the χ^2 test or Fisher's exact test, and noncategorical variables were compared by the Mann–Whitney U test. A p value of <0.05 was considered significant. Concordance between results obtained for whole DBS samples and matched serum samples was established using the Kappa index. All data analysis was performed using program 3.01 (GraphPad InStat® software, San Diego, CA) and MedCalc (version 9.2.1.0, MedCalc Software, Belgium).

4. Results

4.1. Optimisation of HCV AgAb EIAs using DBS

Using the preliminary panel, a 4-fold increase in the DBS initial volume (final volume of 200 μL) gave more appropriate DBS OD values compared to the sera OD values (Table 1). A longer period of DBS sample incubation did not improve the OD values of the DBS samples (Table 2). The sample incubation time recommended by the manufacturer (90 min for the Bio-Rad EIA and 60 min for the Abbott EIA) and a 4-fold increased sample volume without diluent were used.

The CO values were evaluated using the entire population and higher sensitivities and specificities were observed using the ROC curve (CO3) for the Bio-Rad EIA (CO value = 0.287) (Table 3) and the Abbott EIA (CO value = 0.239) (Table 4).

Using a ROC curve, 39 DBS samples of 40 paired HCV AgAb positive serum specimens were detected by both EIAs, while 1 HCV AgAb false positive was observed for the BioRad EIA, and 14 HCV AgAb false positives were detected using the Abbott EIA.

Table 1
Mean optical densities (O.D.) obtained from paired sera and dried blood spot (DBS) samples according to the sample volume used in the assay.

Volume (μL)	Murex HCV AgAb combination EIA (Abbott)			Monolisa™ HCV AgAb ULTRA EIA (Biorad)	
	Diluent volume (μL)	Mean O.D. (nm) Non-reactive sample	Mean O.D. (nm) Reactive sample	Mean O.D. (nm) Non-reactive sample	Mean O.D. (nm) Reactive sample
Sera					
50	50	0.133	2.781	0.044	1.842
DBS					
50	0	0.105	1.282	0.052	0.634
100	0	0.089	1.359	0.066	0.685
150	0	0.105	1.271	0.080	0.735
200	0	0.086	1.224	0.082	0.892
p-Value		0.135	0.999	0.038	0.791

Table 2
Mean optical densities (O.D.) obtained from paired sera and dried blood spots (DBS) according to different incubation periods.

Sample	Murex HCV AgAb combination EIA (Abbott)			Monolisa™ HCV AgAb ULTRA EIA (Biorad)		
	Incubation time	Mean O.D. (nm) Non-reactive sample	Mean O.D. (nm) Reactive sample	Incubation time	Mean O.D. (nm) Non-reactive sample	Mean O.D. (nm) Reactive sample
Sera	60 min	0.133	2.781	90 min	0.044	1.842
DBS	60 min	0.105	1.271	90 min	0.082	0.892
DBS	120 min	0.142	1.462	180 min	0.129	1.192
DBS	18–24 h	0.160	1.035	18–24 h	ND*	ND*

ND*: not done.

Table 3
Accuracy indices for different methods of cut-off absorbance calculation for HCV AgAb detection with DBS samples (Monolisa™ HCV AgAb ULTRA EIA).

Method	Cut-off (nm)	Sensitivity (95% IC)	Specificity (95% IC)	PPV* (95% IC)	NPV* (95% IC)	Accuracy (kappa)
CO1	–	95.00% (83.08–99.39)	100% (98.94–100)	100% (90.75–100)	99.43% (97.94–99.93)	0.971 (0.932–1)
CO2	0.108	97.50% (86.84–99.94)	97.19% (94.90–98.64)	79.59% (65.65–89.76)	99.71% (98.41–99)	0.860 (0.860–1)
CO3	0.287	97.50% (86.84–99.94)	99.71% (98.40–99.99)	100% (90.97–100)	99.97% (98.98–100)	0.986 (0.934–1)

PPV: positive predictive value; NPV: negative predictive value; accuracy: TP + TN/TP + TN + FP + FN CO1: cut-off value determined by the manufacturer; CO2: cut-off value determined by 3 standard deviations above the mean DBS absorbance of HCV seronegative samples (mean + 3 SD); CO3: cut-off value determined by the area under receiver operating characteristic (AUROC) curve analysis for DBS absorbance values.

Excellent agreement among the DBS and sera EIAs was determined by the Kappa index (*k*: 0.972 and 0.817, BioRad and Abbott assays, respectively) (Tables 3 and 4).

4.2. Stability studies

During the period of storage under all conditions, all replicates of the HCV DBS positive samples remained ELISA positive (Figs. 1a and 2a), and the replicates of the HCV DBS negative samples remained negative by the BioRad assay (Fig. 1b). Using the Abbott assay, the DBS negative samples were stable until 60 days of storage (Fig. 2b). The lowest OD value variations were observed at −20 °C, suggesting that this temperature is the best storage condition for DBS.

4.3. Limit of detection

It was possible to detect HCV AgAb among DBS samples using the EIAs and optimised protocol at an estimated viral load of 3.1 IU/mL (dilution $1{:}10^3$).

Reproducibility was obtained by having different operators test the same samples ten times in a single run on two days, using the same lot and the same reagents.[18]

To determine EIA precision, two HCV AgAb reactive DBS samples (dilution 1:10 and 1:1000) were tested 8 times in a single run using both EIAs. A high titre of the HCV AgAb DBS sample (estimated viral load = 310 IU/mL) presented a mean ± SD OD value of 2.417 ± 0.1 for the Bio-Rad EIA and 2.838 ± 0.335 for the Abbott EIA. A low titre of the HCV AgAb DBS sample (estimated viral load 3.1 IU/mL) presented a mean ± SD OD value of

Table 4
Accuracy indices for different methods of cut-off absorbance calculations for HCV AgAb detection with DBS samples (Murex HCV AgAb combination EIA).

Method	Cut-off (nm)	Sensitivity (95% IC)	Specificity (95% IC)	PPV* (95% IC)	NPV* (95% IC)	Accuracy (kappa)
CO1	–	82.50% (67.22–92.66)	97.98% (95.88–99.18)	82.50% (67.22–92.66)	97.98% (95.87–99.18)	0.805 (0.706–0.904)
CO2	0.514	80.00% (64.32–90.94)	99.42% (97.93–99.93)	94.12% (80.32–99.28)	97.73% (95.58–99.01)	0.851 (0.693–0.904)
CO3	0.239	97.50% (86.84–99.94)	95.95% (93.30–97.77)	73.58% (59.72–84.73)	99.97% (98.34–99.99)	0.817 (0.728–0.906)

PPV: positive predictive value; NPV: negative predictive value; accuracy: TP + TN/TP + TN + FP + FN CO1: cut-off value determined by the manufacturer; CO2: cut-off value determined by 3 standard deviations above the mean DBS absorbance of HCV seronegative samples (mean + 3 SD); CO3: cut-off value determined by the area under receiver operating characteristic (AUROC) curve analysis for DBS absorbance values.

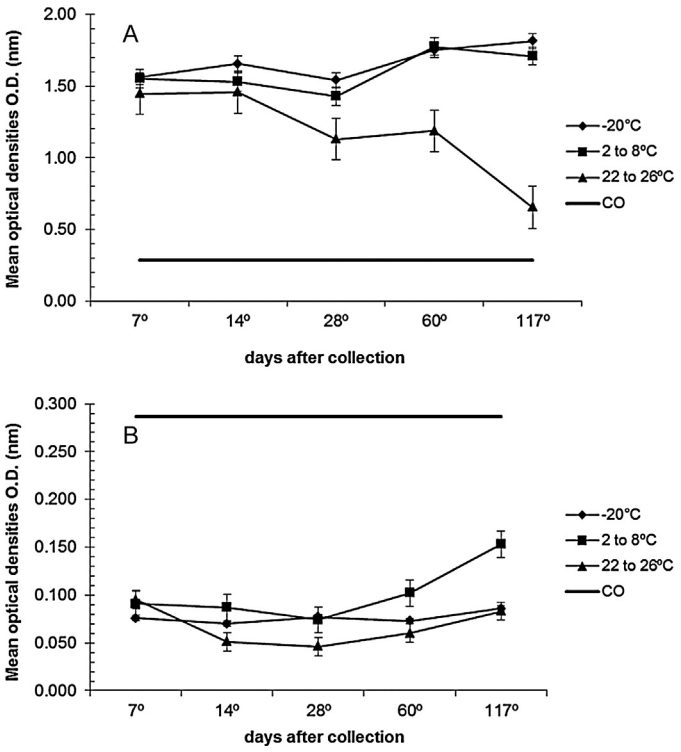

Fig. 1. (a) Stability of reactive HCV Ag–Ab DBS samples obtained during 117-day period at different storage conditions using EIA HCV AgAb: Monolisa™ HCV Ag–Ab ULTRA (Bio-Rad, France). Cut off (CO) value as determined by ROC curve method (0.287 nm). (b) Stability of non-reactive HCV Ag–Ab DBS samples obtained during 117-day period at different storage conditions using EIA HCV AgAb: Monolisa™ HCV Ag–Ab ULTRA (Bio-Rad, France). Cut off (CO) value as determined by ROC curve method (0.287 nm).

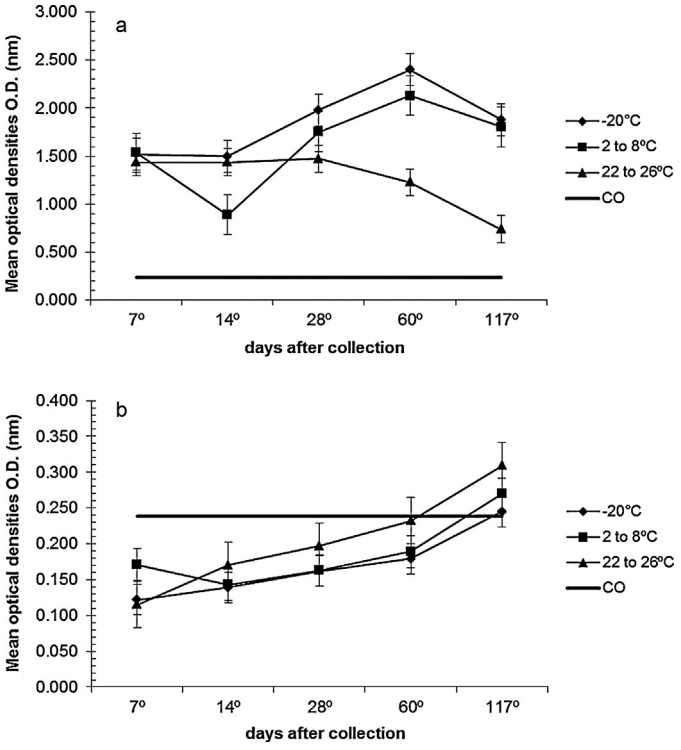

Fig. 2. (a) Stability of reactive HCV Ag–Ab DBS samples obtained during 117-day period at different storage conditions using EIA Murex HCV Ag/Ab (Abbott, South of Africa). Cut off (CO) value as determined by ROC curve method (0.239 nm). (b) Stability of non reactive anti-HCV DBS samples obtained during 117-day period at different storage conditions using EIA Murex HCV Ag/Ab (Abbott, South of Africa). Cut off value as determined by ROC curve method (0.239 nm).

0.633 ± 0.049 using the BioRad EIA and 0.508 ± 0.038 with the Abbott EIA.

For the repetitivity evaluation, high and low titre HCV AgAb DBS samples were tested 20 times in the same run by a single technician using the same reagents, lot and operator. No OD value variation was observed with the Abbott EIA using a high titre sample, while the coefficient of variation (CV) of the OD values was 2.8% using this sample with the BioRad EIA. When the low titre HCV AgAb DBS sample was analysed, the CV was 5.0% for the BioRad EIA and 3.5% for the Abbott EIA. In the reproducibility studies, analyses using high titre HCV AgAb DBS samples presented a CV of 4.0% for the BioRad EIA and 6.7% for the Abbott EIA. For the low titre HCV AgAb DBS sample, the CV was 13.8% for the BioRad EIA and 4.7% for the Abbott EIA (Table 5).

5. Discussion

In this study, it was possible to detect HCV AgAb among dried blood spot samples using commercial EIAs, but some modifications in the protocol should be made. It was necessary to increase the DBS sample volume for HCV AgAb detection, likely due to the low amount of antigens and antibodies present in these samples as compared to the sera samples.[20] Once the DBS is eluted, the antigens and antibodies are probably reduced in these samples, and by increasing the DBS elution volume on the ELISA, the efficiency of the method can be improved.[11,21–23] The sample incubation time did not influence the performance of the assay, as the same as observed for the anti-HCV and HBV markers detection among the DBS samples.[14,15]

Another factor that was evaluated was the cut-off determination, in which the ROC curve gave the highest sensitivities and specificities for HCV AgAb detection using both EIAs, which was similar to results demonstrated for the detection of HBV and anti-HCV in the DBS samples.[14,15] The Biorad EIA performed the best because the sensitivities and specificities were higher than 95% using the cut-off established by the manufacturer. All accuracy indices had good performance, except for the positive predictive values for the Biorad assay using the CO2 values and the Abbott assay using the CO1 and CO3 values. These results were probably due to the low CO values that were used that gave high false positive results or to the inability of the DBS assay to detect HCV AgAb in the samples whose paired sera samples presented low OD values. The present study has documented HCV AgAb detection among DBS samples that can be helpful in identifying individuals at the window period of HCV infection in remote areas.

Regarding DBS stability for HCV AgAb detection, it was possible to obtain accurate results for up to 60 days when the DBS samples were stored in three different conditions (2–8 °C, 20–25 °C and −20 °C). The same effect was observed for anti-HCV detection among the DBS samples in a previous study, and this stability was higher than that reported by Tuaillon et al. in which the samples were stable for 2–12 days before being stored at −20 °C.[12] In this study, −20 °C was the optimum storage condition for extended periods of storage.

Using an optimised assay, a low limit of detection and CV values were observed in the repetitivity and reproducibility studies, which demonstrates the feasibility of the method.

DBS samples can be used for HCV AgAb detection using commercial EIAs, and the Bio-Rad EIA showed the best performance. DBS samples present great stability because HCV AgAb could be detected for two months at three conditions (2–8 °C, 20–25 °C and −20 °C). This method may represent an alternative method for HCV AgAb detection and can overcome the difficulties of blood sampling and management, as evidenced in the screening analysis. This

Table 5
Repetitivity and reproducibility studies for two enzyme immunoassays (EIAs) used for HCVAgAb detection among dried blood spot (DBS) samples with high (10^1) and low viral titres (10^3).

HCV AgAb titre (estimated IU/mL)	Observed optical density of EIA			
	Repetitivity study		Reproducibility study	
	Murex HCV AgAb combination EIA (Abbott) Mean ± SD/CV (%)	Monolisa™ HCV AgAb ULTRA EIA (Biorad) Mean ± SD/CV (%)	Murex HCV AgAb combination EIA (Abbott) Mean ± SD/CV (%)	Monolisa™ HCV AgAb ULTRA EIA (Biorad) Mean ± SD/CV (%)
High titre (310)	3.000 ± 0.000/0.0	2.136 ± 0.059/2.8	2.832 ± 0.189/6.7	2.259 ± 0.090/4.0
Low titre (3.1)	0.466 ± 0.016/3.5	0.453 ± 0.023/5.0	0.423 ± 0.020/4.7	0.507 ± 0.070/13.8

EIA: enzyme immunoassay; SD: standard deviation; CV: coefficient of variation.

method may be especially useful for HCV AgAb detection for individuals living in areas with limited resources.

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Conflict of interest

All authors disclose no actual or potential conflict of interest, including any financial, personal or other relationships with other people or organizations within two years of the beginning of this study that could inappropriately influence the present study.

Ethical approval

The study was approved by Fiocruz (CEP NUMBER 459/08) and HFSE Ethics Committees (CEP NUMBER 000.429).

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